Evidence for involvement of glycoprotein-CD45 phosphatase in reversing glycoprotein-CD3-induced microtubule-associated protein-2 kinase activity in Jurkat T-cells

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Ligation of CD3/TCR on T-cells induces transient activation of lymphoid MAP-2 kinase (MAP-2K), a 43 kDa serine kinase which itself is a substrate of an unidentified tyrosine kinase (pp43). The reversibility of the MAP-2K response agrees with removal of tyrosine phosphates from pp43. Since both activity as well as tyrosine phosphorylation of MAP-2K could be prolonged by Na_3VO_4 , a phosphotyrosine phosphatase inhibitor, we studied the effect of the common CD45 isoform, which is a member of the CD45 phosphatase family, on MAP-2K activity *in vivo* and *in vitro*. We demonstrate the ability of purified CD45 phosphatase to remove tyrosine phosphates from partially purified lymphoid MAP-2K. Utilizing the approach of heterologous receptor aggregation, we also showed that CD45 could inhibit the induction of MAP-2K activity in intact Jurkat cells during CD3 or CD3 + CD4 stimulation. We therefore suggest that this phosphatase may control the activity of lymphoid MAP-2K *in vivo*.

INTRODUCTION

Phosphorylation of proteins on tyrosine residues plays a critical role in the regulation of cellular proliferation and differentiation. In T-lymphocytes, stimulation via the CD3/Ti receptor induces transient appearance of a 43 kDa tyrosine phosphoprotein (pp43) [1–4], which has recently been characterized as a microtubule-associated protein-2 kinase (MAP-2K) [1–3]. T-cell MAP-2K shares many of the characteristics of fibroblast MAP-2K and, as in the latter cell type, needs to be phosphorylated in order to become active during mitogenic stimulation [1–3,5–8]. Since activation of MAP-2K during CD3 ligation is a transient response, it is possible that the rapid reversal of activity may be actively mediated by removing phosphates from this kinase [1,2]. Inasmuch as MAP-2K is a tyrosine phosphoprotein and > 90 % of membrane-associated phosphotyrosine phosphatase activity in T-lymphocytes is contained in the CD45 glycoprotein family [9–11], we looked for a possible role for CD45 in controlling lymphoid MAP-2K activity. We show here that the common CD45 isotype, which is recognized by mAb 9.4 [12], can interfere with induction of CD3-mediated MAP-2K activity and that cross-linking of CD45 to CD3 leads to dephosphorylation of pp43.

MATERIALS AND METHODS

Antibodies

Monoclonal antibodies (mAbs) against CD3 included OKT3 (Ortho, IgG2a) and G19-4 (IgG1) [13]. CD4 ligation was obtained with G17-2 [13], whereas antibody 9.4 [12] was used for ligation of the common CD45 isotype. Co-aggregation of any two or all three of these receptors was achieved by using biotinylated (bio-)

antibodies, followed by avidin cross-linking [13–15]. Affinity-purified anti-phosphotyrosine (APT) antibodies were generated according to the procedure of Kamps & Sefton [16].

Cellular stimulation, lysis and preparation of cellular extracts

Duplicate Jurkat (clone E6-1)-cell aliquots (3×10^6 cells each) were stimulated for various lengths of time with anti-CD3. In certain experiments the effects of anti-CD45 or anti-CD4 on these primary activation pathways were tested by cross-linking biotinylated versions of these antibodies with avidin [3,13–15]. At the termination of culture, the cells were lysed and MAP-2K purified by batch absorption to DEAE as previously described [1–3].

Phosphorylation assays

MAP-2K activity was measured by an '*in vitro*' assay that determines phosphorylation of MAP-2 in the presence of Mn^{2+} as previously described [1–3]. MAP-2 substrate was obtained from rat brain as described [17].

Immunoblotting to detect protein phosphorylation on tyrosine residues

Stimulated or control cell aliquots (2×10^6 cells each) were lysed in hot SDS lysis buffer before proteins were resolved on SDS/10 % polyacrylamide gels and electroblotted on to Immobilon-P membranes [3]. These were sequentially overlaid with APT antiserum (0.25 $\mu\text{g}/\text{ml}$) and ^{125}I -Protein A for autoradiography as previously described [3,15,18].

Studies on intracellular- Ca^{2+} -concentration ($[\text{Ca}^{2+}]_i$) flux by flow cytometry

CD4-positive T-cells were analysed by staining Indo-1-loaded peripheral-blood mononuclear cells with phycoerythrin con-

Abbreviations used: APT, anti-phosphotyrosine; bio-, biotinylated; MAP-2, microtubule-associated protein type 2; MAP-2K, 43 kDa Mn^{2+} -preferring serine kinase with affinity *in vitro* for MAP-2K; MAb, monoclonal antibody; $[\text{Ca}^{2+}]_i$, intracellular Ca^{2+} concentration.

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Fig. 1. Effect of Na_3VO_4 on the kinetics of MAP-2K activation and phosphorylation

(a) Autoradiogram (12 h exposure) showing phosphorylation of MAP-2 substrate during treatment with OKT-3. Duplicate Jurkat cell aliquots were treated with 100 ng of OKT-3/ml in the absence (— or ■) or presence (+ or □) of 0.1 mM- Na_3VO_4 for the time periods indicated. MAP-2K extraction, purification and measurement were performed as previously described [1–3]. MAP-2 bands were sliced from the gel, counted for radioactivity and ^{32}P incorporation was quantified as the mean $\pm$ S.E.M. (graph). Similar results were obtained in a second experiment ($n = 2$). (b) APT immunoblotting of whole-cell lysates showing reactivity of a 43 kDa protein (pp43) that was previously characterized as MAP-2K [3]. Cells were stimulated in the absence or presence of Na_3VO_4 with 100 ng of OKT-3/ml for 2 min (lanes 2 and 8), 5 min (lanes 3 and 9), 15 min (lanes 4 and 10), 30 min (lanes 5 and 11), and 60 min (lanes 6 and 12) respectively. Lanes 1 and 7 are untreated controls. The autoradiogram was exposed for 12 h with an intensifying screen. The specificity of the APT antiserum for phosphotyrosine residues was previously demonstrated [15].

jugates of anti-CD8 (G10-1), anti-CD11b (60.1), anti-class II (HB10a), and anti-CD16 (FC-2) and gating on unstained cells as described [13,18]. Cells were stimulated with biotin-conjugated mAbs and avidin as described [13,14,18]. Cytoplasmic $[\text{Ca}^{2+}]_i$ was measured with an Ortho flow cytometer and peak responses, which occurred within 1 min of cross-linking with avidin, were analysed as described [13,14].

Partial purification of MAP-2K and reaction *in vitro* with CD45 phosphatase

Activated MAP-2K was partially purified over DEAE columns as previously described [1,3]. The effect of CD45 on the state of MAP-2K phosphorylation was tested by adding 1 μg of phosphatase [9,10] (generously supplied by Dr. N. Tonks, Department of Biochemistry, University of Washington, Seattle, WA, U.S.A.) to 50 μl of concentrated DEAE eluate in the presence of 10 mM-dithiothreitol at 37 $^\circ\text{C}$ for 20 min. Phosphoproteins were revealed by APT immunoblotting as described above.

RESULTS

Transient activation of MAP-2K by CD3 is altered by Na_3VO_4

Typical kinetics of MAP-2K activation during ligation of CD3/Ti in Jurkat cells is attainment of a peak of activity at 5 min, followed by rapid decline (Fig. 1a). In the presence of a phosphotyrosine-phosphatase inhibitor, Na_3VO_4 , in the stimulating medium, anti-CD3 induced a response of similar magnitude, but with the difference that MAP-2K activity was sustained for more than 1 h (Fig. 1a). We also obtained soluble extracts from these cells to look for induction of tyrosine phosphokinase activity as detected by APT immunoblotting. The autoradiogram in Fig. 1(b) shows enhanced phosphorylation of a number of substrates inclusive of a 43 kDa substrate (pp43) which, on extensive enrichment, purifies as MAP-2K [3]. Tyrosine phosphorylation of pp43 was maximal 5 min after the addition of anti-CD3 (Fig. 1b, lanes 1–6), but thereupon labelling intensity decreased rapidly and disappeared by 60 min (lanes 4–6). In the presence of Na_3VO_4 , however, tyrosine phosphorylation of pp43 was maintained for up to 60 min (Fig. 1b, lanes 7–12). These results imply an intimate relationship between the stoichiometry of tyrosine phosphorylation and activity of MAP-2K and suggests involvement of both a tyrosine kinase as well as a tyrosine phosphatase.

CD3-induced MAP-2K activity and pp43 tyrosyl phosphorylation is decreased by co-aggregating CD45 with CD3

The most abundant source of membrane-associated tyrosine phosphatase activity in T-lymphocytes resides in the CD45 glycoprotein family [11]. Moreover, CD45 can interfere with CD3 signalling responses [14]. For instance, when cross-linked to CD3, CD45 abrogates the characteristic increase in $[\text{Ca}^{2+}]_i$ (Table 1). Jurkat cells have a relative abundance of the common CD45 isotype as detected by mAb 9.4 during flow cytometry (results not shown). Ligation of CD45 only by biotinylated 9.4 and avidin has no effect on MAP-2K activity (Fig. 2a, bar 3). When MAP-2K activation was induced by a biotinylated anti-CD3 mAb (bio-G19-4) plus avidin (bar 5), it could be shown that heterologous co-aggregation of CD45 abolished this response (bar 6). The effect of anti-CD45 was dose-dependent (insert to Fig. 2b) and could not be mimicked by anti-CD28 or a non-T-cell specific mAb.

It was obligatory for CD45 to be cross-linked to CD3 to demonstrate its inhibitory effect on MAP-2K activity (Fig. 2a, bars 6 and 8). Addition of anti-CD3 and anti-CD45 mAb without avidin cross-linking was followed by the usual stimulation response. Also, co-aggregation of CD45 to CD4, followed

Table 1. Effects of CD4 and CD45 ligation on CD3-induced cytoplasmic Ca^{2+} mobilization in CD4⁺ T-cells

CD4 positive T-cells from peripheral blood were loaded with Indo-1 and cells were stimulated with biotin-conjugated mAbs G19-4 (^{bio}CD3), G17-2 (^{bio}CD4), and 9.4 (^{bio}CD45) for 2 min before addition of avidin. $[\text{Ca}^{2+}]_i$ was measured with an Ortho flow cytometer as described in [13,14]. Identical results were obtained with a second donor's cells ($n = 2$).

Stimulation	Mean peak $[\text{Ca}^{2+}]_i$ (nM)	Cells responding (%)
Unstimulated cells	130	< 5
(^{bio} CD3) + avidin	635	97
(^{bio} CD3 + ^{bio} CD4) + avidin	1346	94
(^{bio} CD3 + ^{bio} CD45) + avidin	312	41
(^{bio} CD3 + ^{bio} CD45 + ^{bio} CD4) + avidin	1296	97
(^{bio} CD3 + ^{bio} CD45 + CD4)* + avidin	289	40

* Note that the anti-CD4 mAb was not conjugated to biotin in this case to demonstrate the importance of CD4 cross-linking to other receptors.

by independent CD3 ligation, failed to inhibit MAP-2K activation (result not shown). However, response reduction was obtained when CD45 was aggregated to both CD3 and CD4 (Fig. 2a, bar 8). This finding is interesting, because under similar conditions (i.e. CD3-CD4-CD45 heteroaggregates), CD4 neutralizes the inhibitory effect of CD45 on $[\text{Ca}^{2+}]_i$ flux (Table 1). The effects of CD45 on induction of $[\text{Ca}^{2+}]_i$ flux and MAP-2K activation by CD3 is therefore discordant in the presence of CD4. In the absence of CD45, CD4 enhances both signals when cross-linked to CD3 (Table 1; Fig. 2a, bar 7).

The kinetics by which CD45 inhibits MAP-2K activity were also studied by introducing the biotinylated anti-CD45 mAb at different times (Fig. 2b). Delaying the introduction of biotinylated anti-CD45 for 2 min after commencing stimulation with biotinylated anti-CD3 plus avidin allowed a MAP-2K response to be registered, but its magnitude was considerably blunted compared with the CD3-only control. In contrast, co-introduction of anti-CD45 at the initiation of anti-CD3 stimulation aborted any response generation (Fig. 2b, inset). These results indicate that CD45 may exert effects on primary activation as well as a MAP-2K response which is already in progress.

Effect of CD45 phosphatase *in vitro* and *in vivo* on phosphorylation of MAP-2K

In Fig. 1(b) we demonstrated that CD3 ligation in Jurkat cells induces tyrosine phosphorylation of pp43 (MAP-2K). In an experiment where phosphorylation of pp43 was obtained through CD3 ligation (Fig. 3a, lanes 2 and 3), cross-linking of CD45 abrogated this event (Fig. 3). This was true regardless of whether CD4 was present (lanes 6 and 7) or absent (lanes 4 and 5) in the heterologous aggregate. In addition to its effect on pp43, CD45 also decreased tyrosine phosphorylation of phosphoproteins of 36, 52 and 58 kDa (lanes 4–7).

MAP-2K was partially purified by DEAE chromatography as previously described [1,3]. Immunoblotting of the peak fraction with APT antiserum revealed the presence of several tyrosine phosphoproteins (Fig. 3b, lane 1) inclusive of pp43 [3]. When the same material was replenished with CD45 phosphatase, the relative abundance of tyrosine phosphate on pp43 was considerably reduced (Fig. 3b, lane 2). There was also an effect on a 36 and 54–56 kDa doublet, but other phosphoproteins were unaffected (lane 2), showing that the effect of CD45 is selective.

Fig. 2. Activation of MAP-2K during CD3-induced phosphorylation is reversed during co-aggregation with CD45

(a) MAP-2K activity as determined by an *in vitro* kinase assay using methods similar to those described in Fig. 1. All mAbs used in this experiment were biotinylated and were added in various combinations to duplicate cellular aliquots for 3 min before the addition of 50 μg of avidin/ml for an additional 5 min. G19-4 (anti-CD3) was used at 0.1 μg/ml, whereas G17-2 (anti-CD4) and 9.4 (anti-CD45) were added to a final concentration of 1 μg/ml. G19-4 instead of OKT-3 was used because it is more suitable for demonstrating the enhancing effect of CD4 (bar 6) ($n = 2$). (b) Kinetics of MAP-2K inhibition by CD45. Biotinylated anti-CD3 (bio-G19-4) was added at 1 μg/ml to duplicate cell aliquots in the absence (—) or presence (---) of 1 μg of bio-9.4/ml at $t = -2$ min. Cellular activation commenced at $t = 0$ min, with the addition of avidin to a final of 50 μg/ml for the time periods indicated. In a third activation protocol, bio-G19-4 was added at $t = -2$, avidin at $t = 0$, whereas bio-9.4 was introduced at $t = +2$ min (— · —). Inset: effect of different doses of bio-9.4 on activation (by bio-CD3 + bio-CD4 + avidin).

Fig. 3. Failure *in vivo* and *in vitro* of tyrosine phosphorylation of pp43 in the presence of CD45

(a) Autoradiogram depicting reactivity of APT antiserum with Jurkat-cell proteins during the following stimulation protocol: lane 1, unstimulated control; lanes 2 and 3, anti-CD3 (G19-4); lanes 4 and 5, anti-CD3 (G19-4)+anti-CD45 (9.4); lanes 6 and 7, anti-CD3+anti-CD4 (G17-2)+anti-CD45 (9.4). All antibodies were biotinylated and added for 3 min before the addition of 50 μ g of avidin/ml for 2 (lanes 2, 4 and 6) or 5 min (lanes 3, 5 and 7) respectively ($n = 2$). (b) Autoradiogram of APT immunoblot. Activated MAP-2K was partially purified over a DEAE column, concentrated, and incubated with highly purified CD45 phosphatase. The phosphoprotein profiles before (lane 1) and after (lane 2) CD45 exposure are shown.

DISCUSSION

We have previously demonstrated that lymphoid MAP-2K is a 43 kDa serine kinase which is activated during mitogenic stimulation of T-lymphocytes [1–3]. Induction of the response via the CD3/Ti receptor is a transient event (Fig. 1), whereas activation with phytohaemagglutinin and the phorbol ester phorbol 12-myristate 13-acetate resulted in prolonged stimulation [1,2]. These differences are principally due to the rate at which MAP-2K activity disappears beyond the peak. Moreover, since MAP-2K activity is intimately dependent on the phosphorylation status of the enzyme [1,3], it suggested that a tyrosine phosphatase may be involved (Fig. 1). This notion was confirmed by coincident appearance/disappearance of MAP-2K activity and phosphorylation of pp43, which is identical with MAP-2K

[3]. Prolonged induction of MAP-2K activity in the presence of Na_3VO_4 further strengthens this notion (Fig. 1a).

Since more than 90% of membrane-located phosphotyrosine phosphatase activity in T-lymphocytes is CD45-associated [11], it was logical to ask whether this glycoprotein family could be involved in the control of MAP-2K activity during CD3 stimulation. Moreover, it has recently been established that purified CD45 phosphatase could directly inactivate MAP-2K purified from fibroblasts [19]. Because it is not known what the ligand(s) for CD45 family is/are and how phosphatase activity is regulated [9,10], we utilized the technique of heterologous receptor aggregation by mAb to demonstrate the effect of CD45 on CD3 signalling in intact lymphocytes [13,14]. Fig. 2 and Table 1 clearly demonstrate the ability of the common CD45 isotype to interfere with MAP-2K activation and the $[\text{Ca}^{2+}]_i$ response. In the performance of these studies it was obligatory for CD45 to be aggregated to CD3 to have an effect on MAP-2K (Fig. 2a). Formation of CD45 homoconjugates, or conjugation of CD45 to CD4 followed by independent CD3 ligation, failed to significantly inhibit the MAP-2K response (results not shown). Although CD4 could increase the MAP-2K response in association with CD3 only, it could not prevent the effect of CD45 on MAP-2K inhibition in CD3/CD4/CD45 heteroaggregates (Fig. 2a). This finding is interesting from the perspective that inhibition of $[\text{Ca}^{2+}]_i$ flux by cross-linking CD45 to CD3 could be opposed by CD4 (Table 1). In the absence of CD45, CD4 enhances $[\text{Ca}^{2+}]_i$ flux (Table 1).

Although exact molecular details of these observations requires further study, it is known that the CD4 is associated with a tyrosine phosphokinase, pp56^{lck} [19,20], and may interact directly with the CD3 receptor [13]. A possible consequence of this interaction is MAP-2K activation, which may explain why experimental coaggregation of CD4 to CD3 can further up-regulate MAP-2K activity [3]. To this end we have previously proposed that MAP-2K may require prior activation of both protein kinase C and pp56^{lck} in order to become active [3]. Proximity of CD4 to CD3 may also be required for activation of inositol-phospholipid-specific phospholipase C, as suggested by the findings that a genistein-sensitive tyrosine phosphokinase is required for inositol-phospholipid turnover and independent aggregation of CD4 reduces the $[\text{Ca}^{2+}]_i$ response to CD3 ligation [3,22]. This may explain the ability of CD4 to enhance $[\text{Ca}^{2+}]_i$ when co-aggregated with CD3 (Table 1). To the contrary, it is possible that CD45 could dephosphorylate phospholipase C with subsequent inhibition of InsP_3 release and $[\text{Ca}^{2+}]_i$ flux (Table 1). The ability of CD4 to interfere with this aspect of CD45 activity, but not its influence on MAP-2K activity, may be the consequence of a direct interaction between pp56^{lck} and CD45 [22–24].

It is therefore against this background that the effect of CD45 on MAP-2K activity must be explored. The first possibility is that CD45 interferes with afferent processes which lead to MAP-2K activation, such as protein kinase C activation. We think, however, that the data in Fig. 2 and Table 1 argue against this possibility. In Table 1 we show that, in the presence of CD4, $\text{PtdIns}(4,5)\text{P}_2$ hydrolysis, as reflected by $[\text{Ca}^{2+}]_i$ flux, is proceeding normally, yet CD45 still inhibits the MAP-2K response (Fig. 2a). Moreover, Fig. 2(b) shows that delayed CD45 introduction could interfere with the MAP-2K response when activation is already in progress. Since this is already beyond the point of maximum InsP_3 hydrolysis, it suggests that CD45 act on a more distal component of the activation cascade. We therefore propose that CD45 may directly dephosphorylate MAP-2K, as is also suggested by the ‘*in vitro*’ experiment (Fig. 3). Although it is not certain at this stage whether lymphoid and fibroblast activities are similar, CD45 phosphatase can directly inactivate both activities [19]. Details about how this putative kinase-phosphatase in-

teraction is mediated in lymphocytes are not available, but apparently it requires the presence of the CD3 receptor (Fig. 2a). By-passing this receptor with phorbol 12-myristate 13-acetate, which also activates MAP-2K, induces a prolonged response which is not affected by CD45 ligation (result not shown).

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